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(54) Title: A SCINTILLATION PROXIMITY ASSAY FOR N-ACETYLGALACTOSAMINYLTRANSFERASE ACTIVITY		
(57) Abstract This invention comprises a scintillation proximity assay designed to assay for the presence of N-acetylgalactosaminyltransferase, also known as GalNAc-transferase. The assay is most conveniently carried out on 96-well microtiterplates. The assay is especially suitable for large volume screens for compounds affecting GalNAc-transferase activity.		

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**A SCINTILLATION PROXIMITY ASSAY FOR
N-ACETYL GALACTOSAMINYLTRANSFERASE ACTIVITY
FIELD OF THE INVENTION**

This invention relates to the field of scintillation proximity assays designed to
5 assay for the presence of UDP-GalNAc:polypeptide, N-
acetylgalactosaminyltransferase, also known as GalNAc-transferase.

INFORMATION DISCLOSURE

Elhammer, Å.P., Poorman, R.A., Brown, E., Maggiora, L.L., Hoogerheide,
J.G., and Kezdy, F.J. (1993) *J. Biol. Chem.* **268**, 10029-10038. Elucidation of the
10 acceptor requirements of the enzyme and construction of acceptor peptides.

Homa, F.L., Hollander, T., Lehman, D.J., Thomsen, D., and Elhammer, Å.P.
(1993) *J. Biol. Chem.* **268**, 12609-12616. Describes the cloning of GalNAc-
transferase, the enzyme used in the assay.

Homa, F.L., Baker, C.A., Thomsen, D.R., and Elhammer, Å.P. (1994) *Prot.*
15 *Expr. Purif.* in press. Describes conversion of the full-length, cloned molecule to a
soluble enzyme and expression and purification of the (soluble) molecule.

J.H. Bertoglio-Matte, US 4,568,649, issued Feb. 4, 1986, "Immediate Ligand
Detection Assay" Describes a SPA based assay for detecting the presence of minute
amounts of an organic reactant in a test sample.

20 O'Connel, B.C., Hagen F.K., and Tabak, L.A. (1992) *J. Biol. Chem.* **267**,
25010-25018. The acceptor specificity of GalNAc-transferase using synthetic
peptides is discussed.

Wang, Y., Agrawal, N., Eckhardt, A.E., Stevens, R.D., and Hill, R.L. (1993) *J.*
Biol. Chem. **268**, 22979-22983. Discusses the acceptor specificity of GalNAc-
25 transferase using synthetic peptides.

Young, J.D., Tsuchiya, D., Sandlin, D.E., and Holroyde, M.J. (1979) *Biochem.*
18, 4444-4448. The original article discussing acceptor specificity of GalNAc-
transferase using synthetic peptides.

Hagopian, A., and Eylar, E.H. (1968) *Arch. Biochem. Biophys.* **128**, 422-433.
30 Background information on the use of synthetic (in this case a modified mucin)
acceptors for the assay of GalNAc-transferase activity.

Hagopian, A., Westall, F.C., Whitehead, J.S., and Eylar, E.H. (1971) *J. Biol.*
Chem. **246**, 2519-2523. Background information on the use of synthetic (in this case
a modified mucin) acceptors for the assay of GalNAc-transferase activity.

35 Sadler, J.E. (1984) in *Biology of Carbohydrates* (Ginsburg, V., & Robbins,

P.W., Eds.) 2, 199-213, John Wiley & Sons, New York. Comprehensive review of O-linked glycosylation.

Schachter, H., and Brockhausen, I. (1992) in *Glycoconjugates* (Allen, H.J., & Kisailu, E.C., Eds.) pp. 263-232, Marcel Dekker, Inc., New York. Comprehensive
5 review of O-linked glycosylation.

BACKGROUND

Mucin type O-glycosidically linked oligosaccharides have been described on a wide variety of protein molecules (Sadler, 1984). These structures constitute essential components in an equally wide variety of biological functions (e.g., Paulson,
10 1989; Jentoft, 1990 and references therein). The initial reaction in the biosynthesis of O-linked oligosaccharides is the transfer of N-acetylgalactosamine from the nucleotide sugar, UDP-N-acetylgalactosamine, to a serine or threonine residue on the acceptor polypeptide. This reaction, which can occur post-translationally, is catalyzed by a GalNAc-transferase enzyme (GalNAcT) called, UDP-GalNAc:polypeptide,N-
15 acetylgalactosaminyltransferase. This is an intracellular membrane bound enzyme believed to be localized in the secretory pathway.

The exact location(s) of GalNAc-transferases in *in vivo* systems is not precisely known. It has been reported that the initial addition of N-acetylgalactosamine to the acceptor protein can take place early (even co-
20 translationally) in the rough endoplasmic reticulum (ER). Other authors have suggested that this reaction is a post-translational event occurring in later ER compartments and/or in the cis region of the Golgi complex (e.g. Hanover *et al.* (1982) *J. Biol. Chem.* 257:10172-10177; Roth (1984) *J. Cell Biol.* 98:399-406; Elhammer and Kornfeld (1984) *J. Cell Biol.* 98:327-331; Tooze *et al.* (1988) *J. Cell*
25 *Biol.* 106:1475-1487; Deschuyteneer *et al.* (1988) *J. Biol. Chem.* 263:2452-2459; Ulmer and Palade (1989) *Proc. Natl. Acad. Sci. (U.S.A.)* 89:663-667; Wertz *et al.* (1989) *J. Virol.* 63:4767-4776; Piller *et al.* (1989) *Eur. J. Biochem.* 183:123-135; Piller *et al.* (1990) *J. Biol. Chem.* 265:9264-9271.

Evidence has also been presented for a model in which transfer of N-acetylgalactosamine to Ser/Thr may occur in several compartments in the secretory
30 pathway, including compartments later than the Golgi complex (Schachter and Brockhausen (1992) in *Glycoconjugates*, Allen and Kisailu, eds., pp. 263-332, Marcel Dekker Inc., New York). Elongation and termination of O-linked oligosaccharides is accomplished by sequential addition of individual monosaccharides by specific
35 transferases (Roseman (1970) *Chem. Phys. Lipids* 5:270-280); current data suggest

that these reactions are localized primarily in the Golgi apparatus (Schachter and Brockhausen, *supra*).

Enzyme-mediated synthesis of O-glycosidically linked oligosaccharides offer significant advantages over the classical synthetic organic pathways, producing very high yields of carbohydrates (e.g., oligosaccharides and/or polysaccharides), under mild conditions in aqueous solutions, and without generating notable amounts of undesired side products. However, an absolute prerequisite for this type of synthesis is the availability of cloned glycosyltransferases.

Endogenous enzymes can be isolated from most eucaryotic sources; however, these proteins are only found in low concentrations, so this is generally a difficult, time consuming procedure, yielding amounts of purified enzymes which are insufficient for *in vitro* synthesis work. Another complication is that the endogenous enzymes invariably are membrane bound, this complicates purification and *in vitro* uses of the enzyme. A cloned enzyme, on the other hand, can usually be expressed as a soluble enzyme with comparative ease.

In light of the considerable value of carbohydrates, there is accordingly a strong felt need for fast and quick assays of GalNAc-transferase.

Assays for GalNAc-transferase activity typically involve incubation of the activity containing preparation with radioactively labeled UDP-GalNAc and either an intact acceptor protein (e.g., basic myelin protein), a fragment(s) of a deglycosylated protein (e.g., various apomucins) or a synthetic peptide (e.g., Hagopian *et al.*, 1971; Hagopian and Eylar, 1968; Young *et al.*, 1979; Wang *et al.*, 1992; Elhammer *et al.*, 1993). The acceptor (sequence) requirements of the enzyme have recently been elucidated to a considerable extent and synthesis of efficient acceptor peptides can be accomplished quite readily (O'Connel *et al.*, 1992; Wang *et al.*, 1993; Elhammer *et al.*, 1993). Following transfer of the radioactive sugar to the polypeptide acceptor, the product is isolated and the amount of enzymatic transfer quantitated by measuring the amount of radioactivity incorporated into the acceptor. Thus, in principle, assays for GalNAc-transferase activity are comparatively straight-forward.

A considerable technical problem associated with these assays, however, is the isolation of the glycosylated reaction product. For peptide acceptors, methods employed to date include chromatography on ion-exchange, size exclusion or reverse phase columns and, for protein acceptors, various (e.g., TCA) precipitation procedures. In the former case, a number of chromatography columns have to be

prepared, equilibrated and developed for each experiment, in the latter, extensive washing procedures have to be carried out in order to reduce background radioactivity. Hence, typical GalNAc-transferase assays (using either acceptor) are both labor intense and time-consuming.

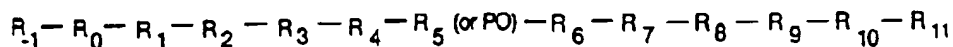
- 5 Here we describe a novel approach for the quantitation of the reaction products in GalNAc-transferase assays that employs fewer steps, takes less time, and is much more suitable than other assays currently available for screening for GalNAc-transferase.

SUMMARY OF THE INVENTION

- 10 The invention comprises a Scintillation Proximity Assay (SPA) for the detection of GalNAc-transferase activity. Embodiments of the assay include: Scintillation Proximity Assay (SPA) beads for the quantitation of incorporated radioactivity; an appropriate receptor interacting with an appropriate ligand produced as a result of reaction with the enzyme, GalNAc-transferase; an
 15 appropriate receptor that is a lectin coated SPA bead and the ligand is an acceptor peptide that is not conjugated; a lectin taken from the group consisting of *Salvia Sclarea*, *Helix Pomatia*, or *Vicia Villosa*; an appropriate receptor that is an antibody coated SPA bead and the ligand is a acceptor peptide that is not conjugated; an antibody that recognizes the Tn antigen; an appropriate receptor that is an antibody
 20 coated SPA bead and the ligand is a modified acceptor peptide fused to an antigenic peptide sequence; an antigenic peptide sequence that is either a portion of MYC (e.g. AEEQKLISEEDLLRKRRQLKHKLEQLRNSC) or FLAG (DYKDDDK); an appropriate receptor that is a avidin or streptavidin coated SPA bead and the ligand is a biotin conjugated acceptor peptide; a biotin conjugated acceptor peptide having a
 25 peptide sequence of at least 3 amino acids where one of the three amino acids is an acceptor peptide that is Ser or Thr; a biotin conjugated acceptor peptide and the peptide sequence may be described as

having the amino acids R_1 to R_{11} , as shown,

30



R_5 (or PO) is the acceptor amino acid and is Ser or Thr;

- 35 R_4 and R_6 are independent and are Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala,

Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp;
 and R_1 - R_3 and R_7 - R_{11} are independent and are, no amino acids, or any of the
 following amino acids, Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr,
 Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp; a biotin conjugated acceptor peptide and
 5 the peptide sequence may be described as

having the amino acids R_1 to R_9 , as shown,

10 $R_1 - R_2 - R_3 - R_4 - R_5 \text{ (or PO)} - R_6 - R_7 - R_8 - R_9$

R_5 (or PO) is the acceptor amino acid and is Ser or Thr;
 R_4 and R_6 are independent and are Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr,
 Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp;
 15 and R_1 - R_3 and R_7 - R_9 are independent and are, no amino acids, or any of the
 following amino acids, Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr,
 Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp; the peptide sequence where the peptides
 are any of the sequences shown in CHART 1; where the amino acids R_1 to R_9 , in the
 acceptor peptide, are any of the following amino acids: Asp, Asn, Glu, Gln, Ser, Gly,
 20 His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp; where the
 amino acids R_1 to R_9 are any of the following amino acids: Ser, Thr, Pro, Ala, or
 Gly; or where the peptide sequences are any of the following,
 RTPPP, RSPPP, PPASTSAPG, or PPASSSAPG.

BRIEF DESCRIPTION OF THE FIGURES

25 **FIGURE 1.** Effect of acceptor conjugate concentration on the formation of
 reaction product.

FIGURE 2. Effect of SPA bead concentration on the recovery of radioactivity
 from the reaction product.

FIGURE 3. Effect of UDP-GalNAc concentration on the formation of reaction
 30 product.

FIGURE 4. Effect of incubation time on the formation of reaction product.

FIGURE 5. Effect of enzyme concentration on the formation of reaction
 product.

ADDITIONAL DESCRIPTION OF THE INVENTION AND DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions and Materials

SPA is Scintillation Proximity Assay. Refer to US Patent 4,568,649 issued 4
5 Feb. 1986, for a complete description of a SPA system. US 4,568,649 incorporated
by reference. GalNAc-transferase is UDP-GalNAc: polypeptide:N-
acetylgalactosaminyltransferase.

(M+H)⁺ is the mass of the ion detected by the MS; this equals the mass of
the molecule (M) plus the mass of one proton (H) and the proton also adds one
10 positive charge (+).

K_m is the so called Michaelis-Menten constant. The numerical value of the
constant provides the substrate concentration at half maximal reaction velocity
($V_{max}/2$).

DPM or dpm is disintegrations per minute. H, h or hr is hour. HPLC is high
15 performance (or pressure) liquid chromatography. TFA is Trifluoro Acetic Acid. The
abbreviation "mU" (milli Units) is a measure of enzyme activity; one U equals 1
nmole GalNAc transferred per minute.

Amino acid residues referred to herein are listed below, they may also be
given either three letter or single letter abbreviations, as follows:

20 Alanine, Ala, A; Arginine, Arg, R; Asparagine, Asn, N; Aspartic acid, Asp,
D; Cystein, Cys, C; Glutamine, Gln, Q; Glutamic Acid, Glu, E; Glycine, Gly, G;
Histidine, His, H; Isoleucine, Ile, I; Leucine, Leu, L; Lysine, Lys, K; Methionine,
Met, M; Phenylalanine, Phe, F; Proline, Pro, P; Serine, Ser, S; Threonine, Thr, T;
Tryptophan, Trp, W; Tyrosine, Tyr, Y; Valine, Val, V; Aspartic acid or Asparagine,
25 Asx, B; Glutamic acid or Glutamine, Glx, Z; Any amino acid, Xaa, X.

All amino acids have a carboxyl group and an amino group. The amino group
of the amino acid is also referred to as the "N-terminus" of the amino acid. The
carboxyl group of an amino acid is also referred to as the "C-terminus" of the amino
acid. The "N-terminus" of an amino acid may form a peptide bond with a carboxyl
30 group of another compound. The carboxyl group that combines with the "N-
terminus" of an amino acid may be the carboxyl group of another amino acid or it
may be from another source. If several amino acids are linked into a polypeptide,
then the polypeptide will have a "free" (unconjugated) N-terminus and a "free" C-
terminus.

35 Peptide Sequences. PPASTSAPG is Pro-Pro-Ala-Ser-Thr-Ser-Ala-Pro-Gly;

PPAdSSdSAPG is Pro-Pro-Ala-D-Ser-Thr-D-Ser-Ala-Pro-Gly; PPASSSAPG is Pro-Pro-Ala-Ser-Ser-Ser-Ala-Pro-Gly; RTPPP is Arg-Thr-Pro-Pro-Pro; RSPPP is Arg-Ser-Pro-Pro-Pro.

For the purposes of this invention, "acceptor" can be any peptide sequence,
5 either free or conjugated, either independent or connected to another compound, that accepts the UDP-GalNAc enzyme.

The words "acceptor peptide" refers to a peptide sequence, such as PPASTSAPG, or to the sequences disclosed in Elhammer, Å.P., Poorman, R.A., Brown, E., Maggiora, L.L., Hoogerheide, J.G., and Kezdy, F.J. (1993) *J. Biol. Chem.*
10 268, 10029-10038, incorporated by reference herein, or to any peptide sequence that accepts the UDP-GalNAc transferase enzyme.

The words "acceptor-conjugate" refers to either an acceptor peptide by itself, in the role of a acceptor-conjugate, or more commonly, it refers to an acceptor peptide, such as PPASTSAPG, that is conjugated to a molecule, such as biotin, that
15 has a high affinity for a compound attached to, or part of, the SPA beads, such as avidin or streptavidin. See, US 4,568,649 for a more complete description of possible acceptor-conjugates. The words "acceptor-biotin-conjugate" would refer to an acceptor-conjugate where biotin was conjugated with the acceptor peptide. The words, "acceptor-biotin-alanine-conjugate" refers to the acceptor peptide conjugated
20 to biotin via alanine, one example of which is biotin- β A β A β A-PPASTSAPG. The alanine acts as a spacer to move the peptide a suitable distance from the biotin. The spacer is not essential but it seems to improve reactivity. Any suitable spacer should have similar effects.

Starting Materials

25 UDP-N-[1-³H]-acetylgalactosamine (8.3 Ci/mmol) and SPA beads may be purchased from Amersham Corp. of Amersham Place, Little Chalfont, England. UDP-N-acetylgalactosamine may be purchased from Sigma Chemical Corp. One cc Bond Elut C₁₈ columns may be purchased from Varian. White Microfluor microtiter plates with round bottom wells may be purchased from Dynatech Laboratories.
30 Purified recombinant, soluble GalNAc-transferase may be prepared as described by Homa *et al.* (1994) *Prot. Expr. Purif.* in press., incorporated by reference.

All other reagents are obtainable from standard sources. The sources provided in the descriptions below represent just one possible source among many that would be known to one skilled in the art.

35 Solid phase peptide synthesis (Barany & Merrifield, 1979) is performed at 0.5

mmole scale utilizing OCH_2 Pam resin, available from Applied Biosystems Inc., Foster City, California, on an Applied Biosystems Inc. 430A Peptide Synthesizer.

Beta-Alanine may be obtained from Advanced Chemtech, Louisville, Kentucky. Other amino acids may be obtained from Applied Biosystems Inc.

5 Biotin in the form of N-hydroxysuccinimide-biotin is available from Pierce Chemical Co.

Utility of the Invention

This invention provides a method to assay GalNAc-transferase activity. The assay is conveniently carried out on 96-well microtiterplates. The assay is suitable
10 for large volume screens for compounds affecting GalNAc-transferase activity. The assay may be made and used as a kit.

Detailed Description of the Invention

This invention comprises an acceptor or conjugated-acceptor, SPA beads and quantitation of incorporated radioactivity. One embodiment comprises a
15 biotinylated acceptor peptide, streptavidin coated SPA beads and quantitation of incorporated radioactivity on a microplate scintillation counter. The assay is ideally suited for experiments involving large series screens for compounds affecting the activity of UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase (GalNAc-transferase).

20 The assay described herein provides a fast, simple and reproducible way of measuring GalNAc-transferase activity. Large numbers of samples can easily be processed and, using the recombinant enzyme, excellent signal-to-noise ratios are easily obtained. The availability of this assay should greatly facilitate screenings for specific GalNAc-transferase inhibitors.

25 Several embodiments of this invention are described:

There are four different, but related, versions of this invention. These different versions are lettered A through D. All the versions utilize SPA beads having appropriate receptors that interact with ligands produced as a result of a reaction with the enzyme, GalNAc-transferase.

30 Version A involves the use of an acceptor peptide, that is not conjugated, and isolation and quantitation of the reaction product, on lectin coated SPA beads. Following GalNAc-transferase catalyzed transfer of ^3H -GalNAc from UDP- ^3H -GalNAc to the acceptor, the glycosylated product is adsorbed to SPA beads coated with a GalNAc-specific lectin. This will bring the radioactive sugar on the reaction
35 product in close enough proximity to the scintillant in the SPA beads to elicit a

signal which can be quantitated in a scintillation detection device. Examples of lectins which specifically binds GalNAc and which could be used for this type of assay are: *Salvia Sclarea*, *Helix Pomatia* and *Vicia Villosa*. Since it is likely that the lectins used for isolation of the reaction product also will have some affinity for the radioactive nucleotide sugar in the assay, this type of assay will probably be applicable primarily to assays using multi-site acceptors.

Version B involves the use of an unconjugated acceptor peptide and isolation and quantitation of the reaction product by antibody coated SPA beads. Following GalNAc-transferase catalyzed transfer of ^3H -GalNAc from UDP- ^3H -GalNAc to the acceptor, the glycosylated product is adsorbed to SPA beads coated with a GalNAc-specific antibody. GalNAc conjugated by an O-glycosidic linkage to a peptide segment is a well documented cancer antigen, the so called Tn antigen, in humans; antibodies specifically recognizing this structure are commercially available. Detection and quantitation of the product generated in this type of assay follows the same principle as in version A.

Version C involves the use of a modified acceptor peptide in which the GalNAc-transferase acceptor sequence is fused to an antigenic peptide sequence. Several such (antigenic) sequences and their corresponding antibodies are described in the literature and some of them, e.g. portions of MYC (e.g. AEEQKLISEEDLLRKRRQLKHKLEQLRNSC) and FLAG (DYKDDDK), are commercially available. Thus, in this type of assay the reaction product is adsorbed on SPA beads coated with an antibody which specifically recognizes the antigenic sequence fused to the acceptor. Detection and quantitation of the product generated in this type of assay follows the same principle as in version A.

Version D involves the use of biotin conjugated acceptor peptides and avidin or streptavidin coated SPA beads. Specific examples and experimental details of this embodiment are provided below.

In one embodiment of a Version D type invention, a previously described (Elhammer *et al.* (1993) *J. Biol. Chem.*, 268, 10029-10038) acceptor peptide, PPASTSAPG, is conjugated to biotin via three β -alanine residues. This makes an acceptor-conjugate, which, in conventional assays, has a K_m comparable to that of the unconjugated peptide and which can be adsorbed to avidin or streptavidin. Activity assays using this peptide in combination with ^3H -labeled UDP-GalNAc (donor substrate) and avidin coated SPA beads result in levels of incorporated radioactivity 10 times greater than background.

An analysis of the time dependency of the enzymatic reaction (using the biotinylated acceptor peptide) yields a K_m of $0.38 \pm 0.12 \mu M$ for UDP-GalNAc. Using, for example, 4 mg of SPA beads, an acceptor concentration of $17 \mu M$, a nucleotide sugar concentration of approximately $0.5 \mu M$ and an enzyme
5 concentration of approximately 7.5 U/ml, the assay demonstrates an approximately linear formation of product for at least 60 minutes.

The assay is most conveniently carried out on 96-well microtiterplates. It is especially suitable for large volume screens for compounds affecting GalNAc-transferase activity.

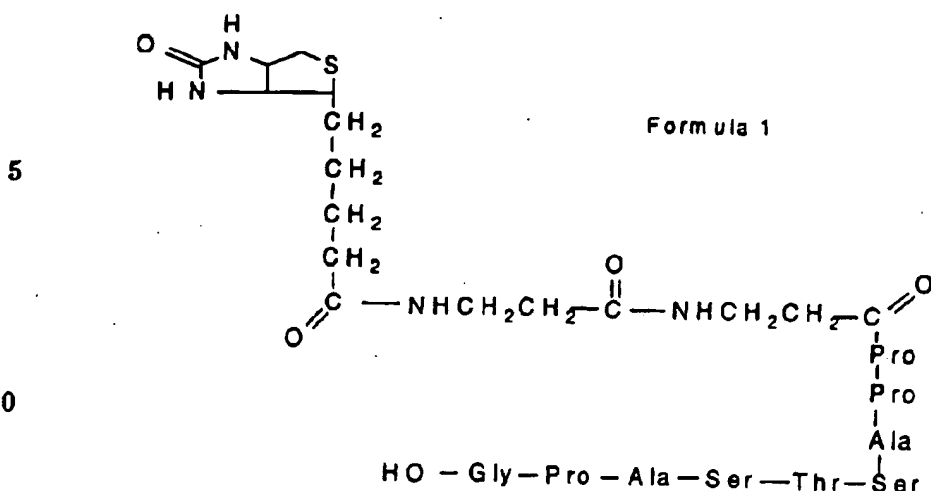
10 *Synthesis of the Biotinylated Acceptor Peptide.*

Solid phase peptide synthesis (Barany & Merrifield, 1979) is performed at 0.5 mmole scale utilizing OCH_2 Pam resin, available from Applied Biosystems Inc., Foster City, California, on an Applied Biosystems Inc. 430A Peptide Synthesizer.

The *t*-butyloxycarbonyl (BOC) group is used as the N-amino protecting group
15 during step-wise synthesis. Tri-functional amino acid side chains are protected with Ser(Bzl) and Thr(Bzl). Each residue is coupled twice, then capped with acetic anhydride before the next cycle of synthesis. Quantitative ninhydrin tests are performed at each cycle of the synthesis.

After removing the N-terminal Boc group in the usual fashion, biotin is
20 attached by treating the peptide-resin in DMF with N-hydroxysuccinimide-biotin, available from Pierce Chemical Co. The biotin-peptide is cleaved from the resin by treatment with HF/anisole(10:1) for 1 hour at -20 to $-5^\circ C$. The peptide resin is titrated with ether, the crude peptide dissolved in 50% acetic acid and the resin is removed by filtration. The filtrate is evaporated to dryness under reduced pressure
25 and lyophilized from glacial acetic acid.

The crude peptide, in the dried lyophilized filtrate, is purified by preparative reverse phase chromatography on a Vydac C-18 column (250 x 22.5 mm) using a water/acetonitrile gradient, with each phase containing 0.1% TFA. Homogenous fractions, as determined by analytical HPLC, are pooled and the acetonitrile
30 evaporated under reduced pressure; an aqueous solution of the pooled fractions is lyophilized. The purified peptide is characterized by time of flight mass spectroscopy which should give the anticipated $(M+H)^+$.



Formula 1 shows the peptide sequence, PPASTSAPG, coupled to biotin.

15 There are numerous peptide sequences that are suitable for this assay. Some sequences are described in Elhammer *et al.* (1993) *J. Biol. Chem.*, 268, 10029-10038, incorporated by reference. Preferred sequences are nine amino acids in length although other lengths are also acceptable. A minimum of 3 amino acids can be recognized by the enzyme. Five amino acids or fewer are less efficient than more

20 than five amino acids. Eight amino acids should perform well. Nine amino acids are preferred. The enzyme seems to recognize 4 amino acids on either side of the acceptor amino acid. Peptides containing ten, eleven, twelve or more amino acids should all function well.

The sequences in CHART I are taken from the Elhammer paper, *id.*, all the

25 peptide sequences in CHART I should work with the assay described herein. CHART I shows 196 glycosylated peptide segments. The glycosylated peptides are listed as enneapeptide (*ennea*, greek: nine) segments with the reactive Ser or Thr in the central position, designated as PO or as R₅. Accordingly, the amino acid side chains toward the NH₂ terminus are designated as the subsites R₁ to R₄ and those

30 toward the COOH terminus as subsites R₆ to R₉, this is shown below.

(acceptor amino acids are Ser or Thr)

(NH₂ terminus side)



(COOH terminus side)

35 R₁ — R₂ — R₃ — R₄ — R₅ (or PO) — R₆ — R₇ — R₈ — R₉

Nine residues is a preferred starting point, with the option that, depending on the results on the selectivity of the subsites, the peptide may be extended or truncated. The sequences in CHART I show that besides the obvious need for Ser or Thr in the PO (or R₅) position, no other subsite has an absolute requirement for any given amino acid.

The following amino acids are all suitable for positions R₁₋₄ and R₆₋₉: Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, Trp.

The enzyme is not highly specific, many sequences will work. Alternative amino acid sequences are also disclosed in the Elhammer paper, *id.* The acceptor amino acid is surrounded preferentially by Ser, Thr, Pro, Ala, and Gly residues, but in no specific order. Both serine and threonine are acceptor amino acids. An ideal glycosylation substrate could consist of the reactive residue surrounded on both sides by Ser, Thr, Pro, Ala, and Gly residues, without any specific order. The hydrogen bonding ability of the peptide backbone should probably remain intact, but the side chains could be varied within large limits without significant deleterious effects.

The amino acids Gly, Ala, Val, and Met are permitted to occur randomly. Perhaps more important is the fact that Asp, Asn, Arg, Tyr, Leu, Phe, Lys, Cys, and Trp occur only at very low frequencies and may decrease the probability of glycosylation.

The following acceptor peptides were synthesized: RTPPP, RSPPP, PPASTSAPG, PPASSSAPG, and PPAdSTdSAPG. The preferred acceptor peptide is PPASTSAPG. Note that the peptide RTPPP has a catalytic efficiency only half that of PPASTSAPG. Further discussion of acceptor efficiencies can be found in Elhammer *et al.* (1993) *J. Biol. Chem.*, 268, 10029-10038.

The Streptavidin Coated SPA Beads

The limited capacity of the streptavidin coated SPA beads used, 115 pmol/mg, (average batch is 110 - 120 pmol/mg) necessitated the use of acceptor concentrations, in the assays, far below saturation.

The optimum acceptor concentration for assays employing 4 mg of the SPA beads, the maximal amount useable in 96-well plates, is shown in FIGURE 1. Maximum incorporation of radioactivity, from the nucleotide sugar onto the acceptor peptide conjugate, occurs when the concentration of acceptor conjugate is approximately 17 μ M. This maximum level of radioactivity incorporation indicates

that low levels of enzyme activity are difficult to detect using this assay method. Comparatively high levels of enzyme activity are needed for positive detection. Typically we use 300mU/assay. Note that other, more conventional assays can easily detect 1mU of enzyme activity; however, other conventional assays cannot
5 conveniently be used for screening purposes.

FIGURE 1 shows the maximum on the incorporation curve (incorporation of radioactive GalNAc from the nucleotide sugar to the acceptor peptide conjugate) which probably reflects saturation of the SPA beads. One would expect a linear increase in radioactivity incorporation up to acceptor saturation if saturation of the
10 SPA beads did not occur.

FIGURE 2 shows the results when different amounts of SPA beads are added to the assay. FIGURE 2 shows that lowering the amount of SPA beads in the assay results in a linear reduction in detected radioactivity, i.e. the amount of SPA beads added is clearly limiting the amount of radioactive product detectable by the assay.
15 However, at 17 μ M acceptor concentration and using 4 mg SPA beads, the assay shows a typical Michaelis-Menten type dependency of the limiting donor concentration.

This typical Michaelis-Menten type dependency of the limiting donor concentration is shown in FIGURE 3. In FIGURE 3, the assay conditions are the
20 same as those described for FIGURE 1. The data in the table below (TABLE 1), taken from FIGURE 3, indicateS the K_m for UDP-GalNAc is calculated to be $K_m = 0.8 \mu$ M.

FIGURE 4 shows time dependency, once again, typical integrated Michaelis-Menten type behavior is indicated by the largely linear shape of the product versus
25 time curve for most of the duration of the reaction. Using the integrated form of the Michaelis-Menten equation and a nonlinear least squares fit method, we calculate $K_m = 0.38 \pm 0.12 \mu$ M for UDP-GalNAc under our experimental conditions. The agreement of the experimental data and the theoretical curve based on the best fit parameters of the Michaelis-Menten equation indicate that the reaction is
30 uncomplicated by substrate inhibition or by enzyme decomposition. The slight curvature of the reaction during the first 60 minutes shows that the quantity of enzyme used in this assay is very probably an upper limit where linearity of the assay velocity versus enzyme concentration still obtains.

FIGURE 4 shows the results of another aspect of this assay, the amount of
35 enzyme used in the assay. The addition of increasing amounts of enzyme results in

a linear increase in product formation up to approximately 600 mU. Our typical assay uses approximately 300 mU of enzyme.

Conventional assays for the evaluation of the biotinylated acceptor peptide were carried out in order to make comparisons with the SPA assay. These assays were carried out as described by Homa *et al.* (1994). A one cc Bond Elut column may be used for isolation of the reaction product. See, Homa, F.L., Baker, C.A., Thomsen, D.R., and Elhammer, Å.P. (1994) *Prot. Expr. Purif. in press.*, incorporated herein by reference. The SPA based assay is carried out on 96-well microtiter plates.

One ordinarily skilled in the art should be able to practice the invention without further instruction. The following examples are provided to further illustrate and not limit the invention. While the invention will now be described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes, obvious to one skilled in the art, may be made without departing from the invention.

EXAMPLES

EXAMPLE 1. A typical reaction. Reaction mixtures will vary depending on the specific experiment. See the figures and tables for the specific concentrations of various components. A typical reaction mixture contains the following components in a final volume of 40 μ l: 2 μ mol imidazole, pH 7.2, 0.4 μ mol MnCl_2 , 19.2 pmol (135,000 cpm) UDP-[^3H]-GalNAc, 0.7 nmol acceptor peptide (biotin- β A β A β A-PPASTSAPG) and approximately 300 mU of enzyme.

The reaction is incubated at 37°C for 30 minutes, then the reaction is quenched by the addition of 10 μ l 0.5 M EDTA. One hundred microliters of a 40 mg/ml suspension of streptavidin SPA beads in PBS containing 20% glycerol is added to each reaction mixture (well on microtiter plate) and the plate is incubated at room temperature on an orbital shaker for 2 hours before the incorporated radioactivity is counted on a microplate scintillation counter.

Assays containing this peptide, in up to saturating concentrations, (and using reverse-phase columns for isolation of the reaction product) demonstrate that the K_m for this acceptor is similar to the K_m previously determined for the unbiotinylated peptide, 1.7 mM *vs.* 6.5 mM (Elhammer *et al.*, 1993). Thus, the addition of biotin to the NH_2 -terminus of PPASTSAPG does not adversely affect the efficiency of this acceptor. In fact, the slightly lower K_m (*vs.* the unbiotinylated peptide) indicates that biotinylation may improve acceptor efficiency.

EXAMPLE 2. FIGURE 1. Effect of acceptor conjugate concentration on the formation of reaction product. Assays were carried out in a 96-well microtiter plate and contained 2 μmol imidazole, pH 7.2, 0.4 μmol MnCl_2 , 19.2 pmol UDP- ^3H -GalNAc, approximately 300 mU of enzyme and 0 to 30 μmol acceptor peptide, in a total volume of 40 μl . After incubation for 1 hour at 37°, the reaction was stopped by the addition of 10 μl of 0.5 M EDTA. One hundred μl of a 40 mg/ml suspension of streptavidin SPA beads were added and, following incubation for two hours at room temperature, the radioactivity in the wells was counted in a Top Count scintillation counter. See FIGURE 1 for results. The solid diagonal line, in FIGURE 1, to the left of the vertical line, is a linear least squares fit of the data obtained when the acceptor peptide concentration was 13 μM and lower. The horizontal solid line was obtained by calculating the mean DPM when the acceptor peptide concentration was 17 μM and higher.

EXAMPLE 3. FIGURE 2. Effect of SPA bead concentration on the recovery of radioactivity from the reaction product. Assay conditions were as described for EXAMPLE 2. The acceptor conjugate concentration was 17 μM and the amount of SPA beads added to the assay was varied from 0 to 100 μl (4 mg). The solid line is a linear least squares fit to the data.

EXAMPLE 4. FIGURE 3. Effect of UDP-GalNAc concentration on the formation of reaction product. Assay conditions are the same as those described for EXAMPLE 2. The acceptor conjugate concentration was 17 μM . The amount of UDP- ^3H -GalNAc varies from 0 to 28.8 pmol. The solid curve is a nonlinear least squares fit to the Michaelis-Menten equation where $V_{\text{max}} = 37.8 \pm 4.6 \times 10^3 \text{ DPM/h}$ and $K_m = .8 \pm .2 \mu\text{M}$.

EXAMPLE 5. FIGURE 4. Effect of incubation time on the formation of reaction product. Assay conditions are the same as those described in EXAMPLE 2; acceptor conjugate concentration was 17 μM . The incubation time was varied from 0-8 hours. The solid curve is a nonlinear least squares fit to an integrated form of the Michaelis-Menten equation, where the K_m for GalNAc was found to be $0.38 \pm 0.12 \mu\text{M}$.

EXAMPLE 6. FIGURE 5. Effect of enzyme concentration on the formation of reaction product. Assay conditions are the same as those described in EXAMPLE 2. Acceptor conjugate concentration is 17 μM . The enzyme concentration in the assays was varied from 0-1500 mU. See, FIGURE 5. The solid line is a linear least squares fit to the data.

CHART I follows. **CHART I** shows various amino acid substitutions for possible peptide sequences for the acceptor peptide. The chart show positions corresponding to positions R_{1-4} - (PO or R_5) - R_{6-9} . The R_5 (or PO) position is always Ser or Thr.

5

CHART I

	PPTP-S-PSTP	TTSI-T-SDPK	PSFN-T-PSTR	GPVV-T-AQYE
5	TPSP-S-TPPT	ESPS-T-SEAL	-S-T-GS-	AVTG-S-EPGL
	TPPP-T-SGPT	VAVP-T-TSA-	LVST-S-EPLS	DVNC-S-GPTP
	VTPR-T-PPPS	TTTS-S-SVSK	-S-T-TAVQ	SLGP-S-KETH
	CPVP-S-TPPT	QTPT-S-GEPL	LPGV-T-GTSA	PIAG-T-SDLS
	APAR-S-PSPS	VPGG-S-ATPQ	PEAT-T-ESII	-Q-T-IATG
10	SPSP-S-TQPW	MHTT-T-IAEP	TTSS-S-VSKS	KSYI-S-SQTN
	SGEP-T-STPT	GGTI-T-TNSP	TMHT-T-TIAE	-L-S-TTEV
	PATW-T-VPPP	PGLP-S-TGVS	GEQG-S-ATPG	DPGM-S-GWPD
	APPP-S-LPSP	PVTI-T-NPAT	VTGT-S-AVTG	ISSQ-T-NDTH
	NSAP-T-SSST	KPSA-T-SPGV	KMYT-T-SITS	HQIS-S-KLPT
15	MHTT-T-SSSV	ASAS-T-TMHT	ATPG-S-TTGR	THGL-S-ATIA
	TPHA-T-SHPA	GGSA-T-PQQP	STGV-S-GLPG	VSEI-S-VRIV
	SSVP-T-AQPQ	PSLP-S-PSRL	GLPS-T-GVSG	SDLS-T-ITSA
	PAPA-T-EPTV	ATAA-T-AATA	AQPL-T-ENPR	QVLL-S-NPTS
	-SKP-T-CPPP	TPSP-S-CCHP	LAKA-T-TAPA	ALSE-S-TTQL
20	PGMA-S-ASTT	---T-ETPV	MLPF-T-PNSE	QGSA-T-PGNV
	TVEP-T-PAPA	GVTG-T-SAVT	VPQE-T-PHAT	AHEV-S-EISV
	AMHT-T-TSSS	TAPA-T-TRNT	TSDL-S-TITS	AWPL-S-LEPD
	STIT-S-AATP	FTPN-S-ESPS	VTMA-T-GSLG	SEPL-S-SKMY
	QTIA-T-GSPP	NPAT-S-SAVA	LPGP-S-DTPI	VSLE-T-SKGT
25	HTTT-S-SSVS	SEST-T-QLPG	ETPV-T-GEQG	NATV-T-AGKP
	PPTP-S-PSCC	PVLP-T-QSAH	LPGS-T---	IIIP-T-INTI
	ESII-T-STPE	WSTR-S-PNST	SSPL-S-TERM	--R-S-AGAG
	ELAP-T-APPE	AQAS-S-VPTA	MYTT-S-ITSD	FVHV-S-ESFP
	TSAA-T-PTFT	PHAT-S-HPAV	GTSA-T-VSLE	-RS-S-VPGG
30	IITS-T-PETP	LPSP-S-RLPG	IATG-S-PPIA	DSQQ-T-AR-
	PLVS-T-SEPL	GPVP-T-PPDN	-LS-T-TEVA	AGAG-T-AGVD
	-IA-T-PLPP	-PGG-S-SEPK	-STG-S---	AGFI-S-TEDP
	PSPS-T-QPWE	-ST-T-AVQT	EQPL-T-ENPR	REYT-S-ARS-
	VQTP-T-SGEP	AKAT-T-APAT	ISVR-T-VYPP	EPLS-S-KMYT
35	PGAL-S-ESTT	-MW-S-TRSP	LSTY-S-SIAT	VQKE-T-GVPE
	LSTI-T-SAAT	TPTF-T-TEQD	TYAA-T-PRAH	EALS-T-YSSI
	SESP-S-TSEA	VAMH-T-TTSS	SGVA-S-DPPV	NLPN-T-MTML
	PNSE-S-PSTS	KAQA-S-SVPT	GLPG-S-T-	IKNT-T-AVVQ
	GTIT-T-NSPE	STYS-S-IATV	PDAA-S-AAPL	-RFS-S-AGIP
40	SAST-T-MHTT	TAVQ-T-PTSG	ATEP-T-VDSV	KADS-T-GDQT
	AVPT-T-SA-	STTV-S-LPHS	VSNA-T-VTAG	SKLP-T-QAGF
	SPST-S-EALS	DSVV-T-PEAT	PHQI-S-SKLP	AGVD-S-QQTA
	GIPA-T-PGTS	PGST-T-GR-	PNTM-T-MLPF	LFPK-S-SGVA
	TSAV-T-GSEP	LSES-T-TQLP	-V-T-LSPK	YQEV-S-IKMS
45	DSSS-S-KAPP	TPGS-T-TGR-	SKMY-T-TSIT	-RF-S-SAGI
	TNPA-T-SSAV	APAT-T-RNTG	EVRP-T-SAVA	REDP-S-GTMY
	MASA-S-TTMH	GLSA-T-IATS	-LST-T-EVAM	EEEG-S-GGGQ
	PSAT-S-PQVM	LPPS-T-SINE	DNTV-T-SKPL	YYNQ-S-EAGS
	SSIA-T-VPVT	MATG-S-LGPS	SEAL-S-TYSS	RFQD-S-SSSK
50	ARSP-S-PSTQ	GFIS-T-EDPS	SIKM-S-SVPQ	PENF-S-FPDD
	PATS-S-AVAS	-R-S-SVPG	-MWS-T-RSPN	GFNM-S-LLEN
	EPLV-S-TSEP	TTMH-T-TTIA	GPVV-T-AQYE	NVYR-S-HLFF

CLAIMS

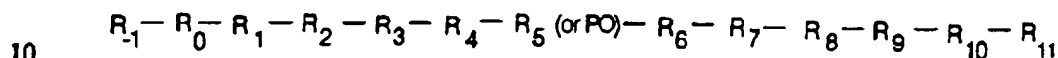
We claim:

1. A Scintillation Proximity Assay (SPA) for the detection of GalNAc-transferase activity.
- 5 2. An assay of claim 1 comprising Scintillation Proximity Assay (SPA) beads for the quantitation of incorporated radioactivity.
3. An assay of claim 2 comprising an appropriate receptor interacting with an
10 appropriate ligand produced as a result of reaction with the enzyme, GalNAc-transferase.
4. An assay of claim 3 comprising an appropriate receptor that is a lectin coated SPA bead and the ligand is an acceptor peptide that is not conjugated.
- 15 5. An assay of claim 4 comprising a lectin taken from the group consisting of *Salvia Sclarea*, *Helix Pomatia*, or *Vicia Villosa*.
6. An assay of claim 3 comprising an appropriate receptor that is an antibody
20 coated SPA bead and the ligand is a acceptor peptide that is not conjugated.
7. An assay of claim 6 comprising an antibody that recognizes the Tn antigen.
8. An assay of claim 3 comprising an appropriate receptor that is an antibody
25 coated SPA bead and the ligand is a modified acceptor peptide fused to an antigenic peptide sequence.
9. An assay of claim 8 comprising an antigenic peptide sequence that is either a portion of MYC (e.g. AEEQKLISEEDLLRKRRERQLKHKLEQLRNSC) or FLAG
30 (DYKDDDK).
10. An assay of claim 3 comprising an appropriate receptor that is a avidin or streptavidin coated SPA bead and the ligand is a biotin conjugated acceptor peptide.

11. An assay of claim 10 comprising a biotin conjugated acceptor peptide having a peptide sequence of at least 3 amino acids where one of the three amino acids is an acceptor peptide that is Ser or Thr.

5 12. An assay of claim 11 comprising a biotin conjugated acceptor peptide and the peptide sequence may be described as

having the amino acids R_1 to R_{11} , as shown,



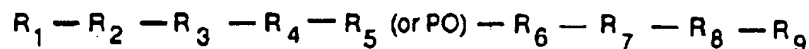
R_5 (or PO) is the acceptor amino acid and is Ser or Thr;

R_4 and R_6 are independent and are Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp;

15 and $R_1 - R_3$ and $R_7 - R_{11}$ are independent and are, no amino acids, or any of the following amino acids, Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp.

13. An assay of claim 12 comprising a biotin conjugated acceptor peptide and the
20 peptide sequence may be described as

having the amino acids R_1 to R_9 , as shown,



25

R_5 (or PO) is the acceptor amino acid and is Ser or Thr;

R_4 and R_6 are independent and are Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp;

30 and $R_1 - R_3$ and $R_7 - R_9$ are independent and are, no amino acids, or any of the following amino acids, Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp.

14. An assay of claim 13 where the peptide sequence are any of the sequences shown in CHART I.

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15. An assay of claim 14 where the amino acids R_1 to R_9 , in the acceptor peptide, are any of the following amino acids: Asp, Asn, Glu, Gln, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, Lys, Cys, or Trp.
- 5 16. An assay of claim 14 where the amino acids R_1 to R_9 are any of the following amino acids: Ser, Thr, Pro, Ala, or Gly.
17. An assay of claim 12 where the peptide sequences are any of the following, RTPPP, RSPPP, PPASTSAPG, or PPASSSAPG.
- 10 18. An assay of claim 16 where the peptide sequence is, PPASTSAPG.
19. An assay of claim 13 where there are 9 amino acids in the peptide sequence.
- 15 20. An assay of claim 19 where the amino acids are comprised of Ser, Thr, Pro, Ala, or Gly.
21. An assay of claim 20 where the peptide sequence is PPASTSAPG or PPASSSAPG.

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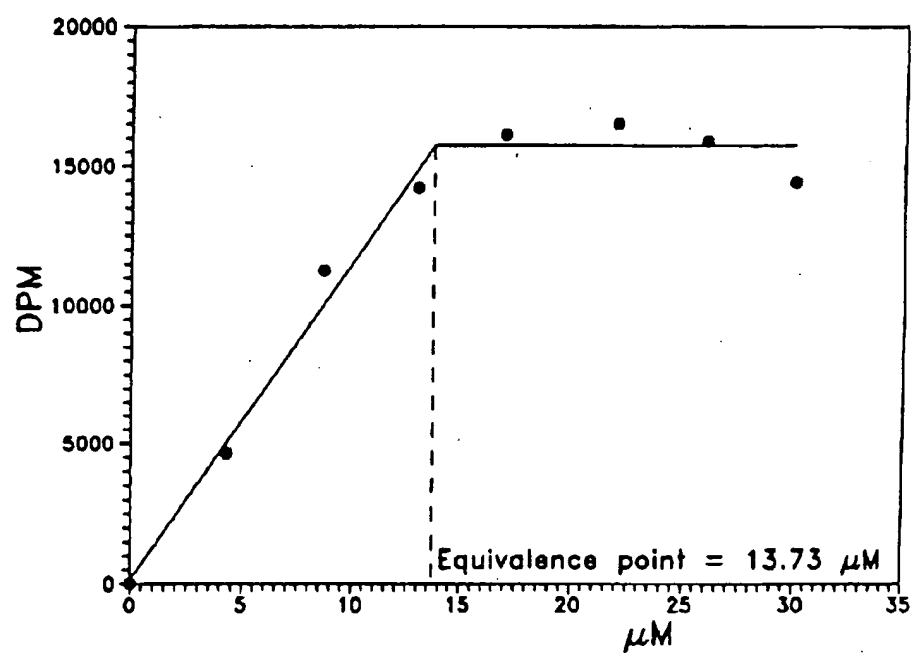
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FIGURE 1

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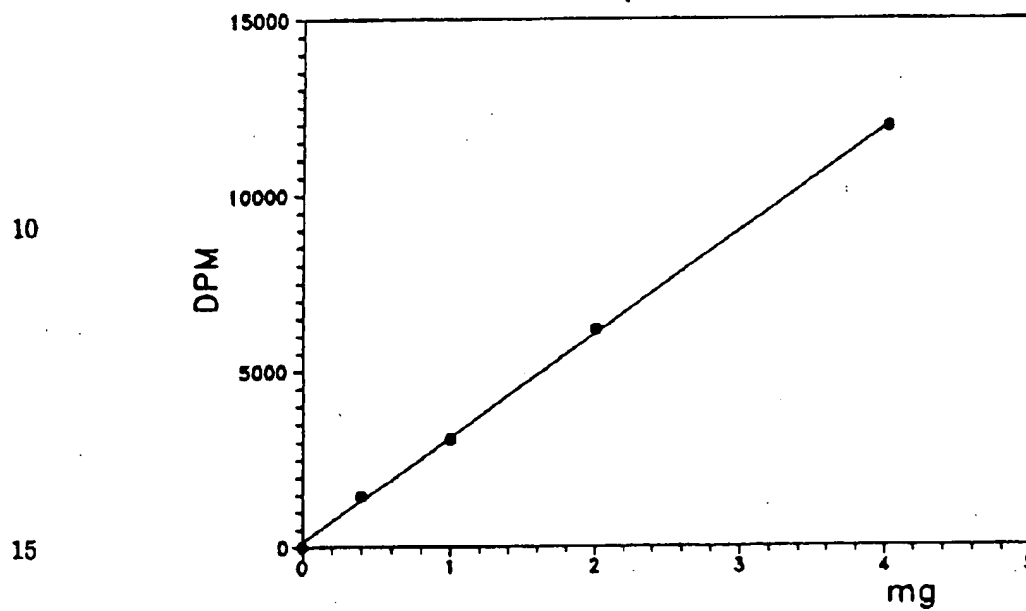
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FIGURE 2

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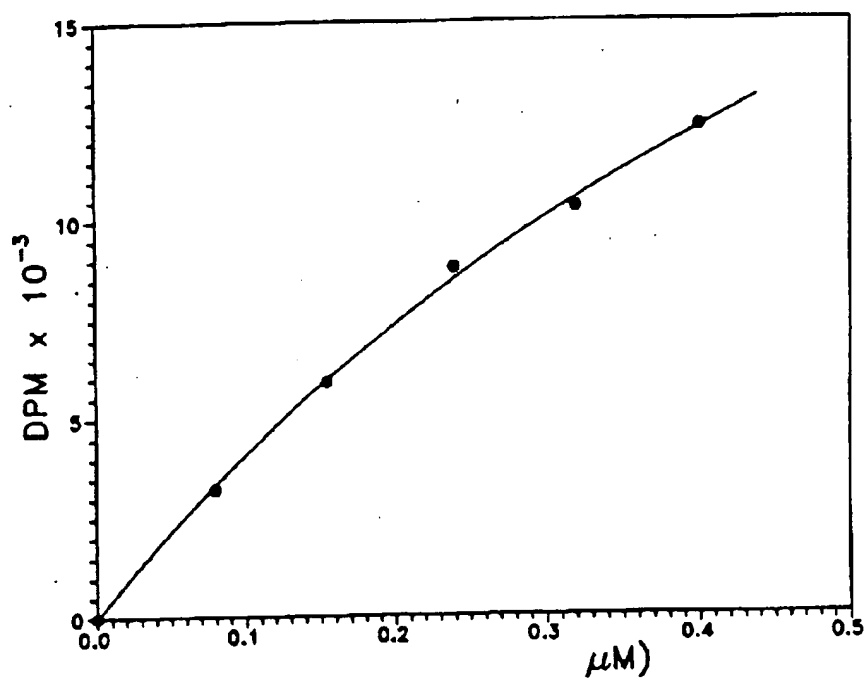
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5 FIGURE 3

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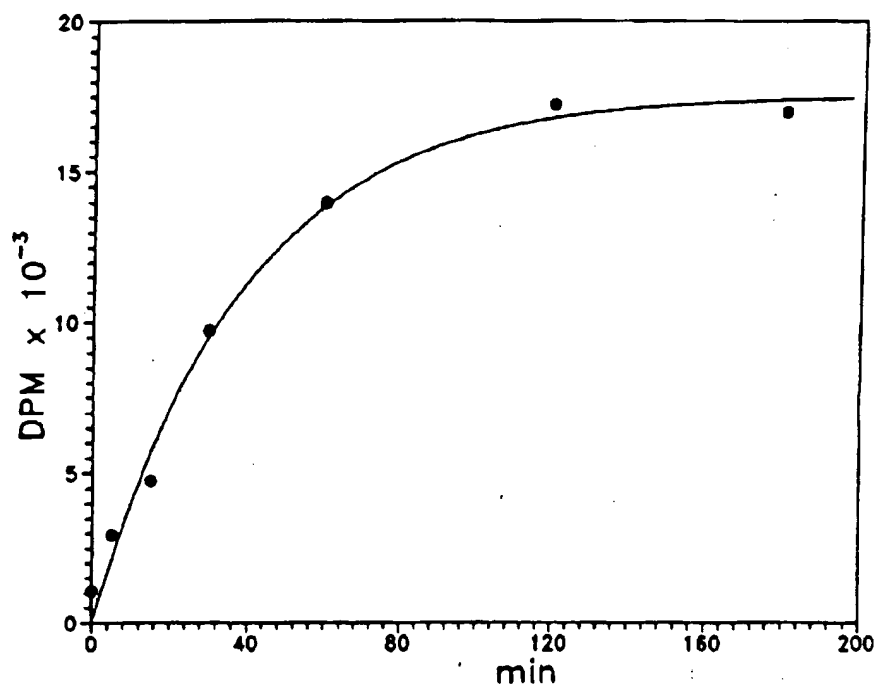
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FIGURE 4

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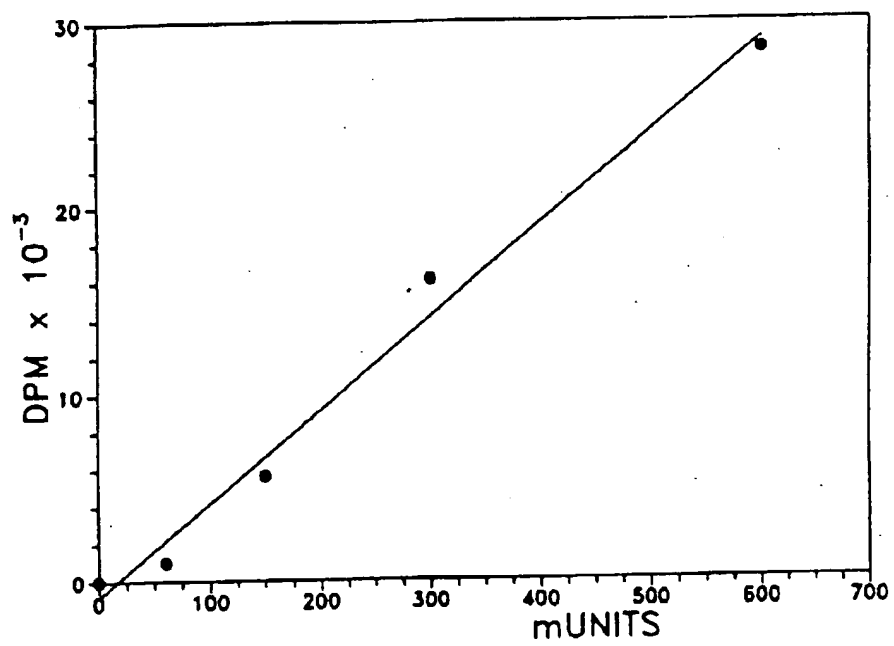
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5 FIGURE 5

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 95/13483

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/48 G01N33/546

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP.A,0 387 875 (SUMITOMO SEIKA CHEMICALS CO., LTD) 19 September 1990 see the whole document ---	1-21
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 14, 15 May 1993 pages 10029-10038. A. P. ELHAMMER ET AL. 'The specificity of UDP-GalNAc:polypeptide N-Acetylgalactosaminyltransferase as inferred from a Database of in vivo substrates and from the in vitro glycosylation of proteins and peptides.' cited in the application see the whole document --- -/--	1-21

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Date of the actual completion of the international search

29 March 1996

Date of mailing of the international search report

23.04.96

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 95/13483

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,4 568 649 (J. H. BERTOGLIO-MATTE) 4 February 1986 cited in the application ---	1-21
P,Y	EP,A,0 656 422 (AMERSHAM INTERNATIONAL PLC) 7 June 1995 see page 4 ---	
P,A	WO,A,94 26906 (THE UPJOHN COMPANY) 24 November 1994 -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/13483

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-387875	19-09-90	DE-D- 69022867 JP-A- 3015761	16-11-95 24-01-91
US-A-4568649	04-02-86	NONE	
EP-A-656422	07-06-95	NONE	
WO-A-9426906	24-11-94	AU-B- 6663294 EP-A- 0698103	12-12-94 28-02-96

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